Dog and human acid β -D-galactosidases are structurally similar

Jeffrey J. HUBERT and John S. O'BRIEN*

Department of Neurosciences, M008, School of Medicine, University of California, San Diego, La Jolla, CA 92093, U.S.A.

(Received 13 December 1982/Accepted 13 April 1983)

The purification of dog liver acid β -galactosidase is described. The dog enzyme migrated as a single major band on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, with a molecular weight of 60 000. Antiserum raised against purified human liver acid β -galactosidase cross-reacted with β -galactosidase from dog liver, but not with those from cat liver or *Escherichia coli*. Tryptic peptide maps of the dog and human acid β -galactosidases indicate that 21 of the 24 peptides observed were homologous; a similar result was obtained after chymotryptic peptide mapping. We conclude that dog and human acid β -galactosidases are structurally similar, and that canine G_{M1} gangliosidosis (acid β -galactosidase deficiency) is an excellent model for the same disease in man.

The human disease G_{M1} gangliosidosis is transmitted as an autosomal recessive trait and involves the accumulation of galactose-containing oligosaccharides and ganglioside G_{M1} in visceral organs and brain as a consequence of a profound deficiency of activity of lysosomal acid β -D-galactosidase (O'Brien, 1978). Canine G_{M1} gangliosidosis has been described in mixed-breed beagles (Read *et al.*, 1976) and resembles the human disease in the following respects: the canine disorder is transmitted as an autosomal recessive trait; ganglioside-G_{M1} accumulation occurs in the nervous system; massive visceral accumulation of galactose-containing oligosaccharides occurs (Warner & O'Brien, 1982), and β -galactosidase activity is profoundly deficient, to values about 4% of normal (Rittmann *et al.*, 1980). Clinically and pathologically the canine disorder resembles human juvenile G_{M1} gangliosidosis (O'Brien, 1978).

It is important to determine whether the dog enzyme is structurally similar to human acid β -galactosidase. To this end, we have performed structural studies on highly purified dog liver acid β -galactosidase and compared the results with those obtained on the human enzyme. Our results indicate that the dog enzyme is structurally similar to the human enzyme.

Experimental

Materials

Fresh frozen dog livers were obtained from Pel-Freeze Inc. (Rogers, AK, U.S.A.); concanavalin A-Sepharose 4B was from Pharmacia (Uppsala, Sweden); D-galactono- γ -lactone and 4-methylumbelliferyl β -D-galactopyranoside were from Koch-Light Laboratories (Colnbrook, Bucks., U.K.); D-galactose and *Escherichia coli* β -galactosidase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); staphylococcal A protein was from Calbiochem Behring (La Jolla, CA, U.S.A.); chemicals for polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); Amicon ultrafiltration devices and Diaflo PM-10 membranes were obtained from Amicon Corp. (Lexington, MA, U.S.A.). Sepharose 4B-6-aminohexyl 1-thio- β -D-galactopyranoside columns were prepared by coupling 6-aminohexyl 1-thio- β -D-galactopyranoside to Sepharose 4B as described previously (Frost *et al.*, 1978). Samples of purified acid β -galactosidase from human liver and cat liver were available for use, prepared as described previously (Frost *et al.*, 1978; Holmes & O'Brien, 1979).

Methods

Purification of dog liver acid β -galactosidase. All procedures were performed at 0–4°C unless otherwise specified. Acid β -D-galactosidase was assayed as described by Norden *et al.* (1974), with

Abbreviation used: SDS, sodium dodecyl sulphate.

* To whom reprint requests should be addressed.

4-methylumbelliferyl β -D-galactopyranoside as substrate, and protein was determined by the method of Lowry *et al.* (1951). In a typical experiment, 1 kg portions of pooled frozen dog liver were cut into small pieces and homogenized at 4°C in a Waring blender for 1 min at low speed with 3 litres of 5 mM-sodium phosphate buffer, pH 7.0, containing 100 mM-NaCl and 0.02% NaN_3 . The homogenate was centrifuged for 90 min at 24 000 g and the resultant supernatant was chromatographed on a column (2 cm $\times$ 16 cm; 200 ml) of concanavalin A-Sepharose as previously described (Frost *et al.*, 1978); β -galactosidase was eluted with α -methylmannoside. The α -methyl D-mannoside eluate was concentrated to a protein concentration of 18 mg/ml by ultrafiltration on an Amicon concentrator equipped with a UM 5 Diaflo membrane. The concentrate was dialysed for 16 h against two changes of 4 litres of 10 mM-sodium phosphate, pH 7.0, containing 100 mM-NaCl and 0.04% NaN_3 . The dialysed preparation was then centrifuged to remove particulate matter, at 24 000 g for 30 min.

Acid β -galactosidase was then chromatographed on Sepharose 4B-6-aminohexyl 1-thio- β -D-galactopyranoside as previously described (Frost *et al.*, 1978), except for the following changes. The pH of the enzyme solution was adjusted to 4.0 instead of 5.4, since it was found that the dog enzyme did not adsorb well to the column at the higher pH. In all other aspects, elution from the thiogalactoside affinity column with D-galactose and D-galactonolactone and final preparation of the purified enzyme were the same as that for the human enzyme (Frost *et al.*, 1978).

Polyacrylamide-gel electrophoresis. Samples of purified dog β -galactosidase were subjected to polyacrylamide-gel electrophoresis on 12.5% (w/v)-acrylamide slab gels in the presence of SDS as described by Laemmli (1970). The following proteins were selected as molecular-weight standards: phosphorylase *a* (mol.wt. 9.4×10^4), bovine serum albumin (6.8×10^4), goat albumin (4.3×10^4), soya-bean agglutinin (3.0×10^4) and human acid β -galactosidase (6.5×10^4). A portion (10 μ g) of each protein standard was made 1% (w/v) in SDS and 5% (v/v) in 2-mercaptoethanol, and these were heated at 80°C for 5 min. Samples of dog β -galactosidase were made 1% (w/v) in SDS and then (a) subjected directly to electrophoresis, or (b) made 5% (v/v) in 2-mercaptoethanol and subjected to electrophoresis, or (c) made 5% (v/v) in 2-mercaptoethanol and heated at 80°C for 5 min. After electrophoresis at 15 mA for 4 h at 22°C, gels were fixed and stained for 17 h in 0.2% Coomassie Brilliant Blue G250 and destained in 10% acetic acid/4% methanol as described by Weber & Osborn (1969). Molecular-weight estimations on the dog enzyme were made by comparison of the log of the

molecular weight versus migration of the protein with respect to Bromophenol Blue in the same manner as for determinations on the human enzyme (Frost *et al.*, 1978).

Immunoprecipitation. Antibody previously raised against homogeneous human liver β -galactosidase (A2 plus A3) (Frost *et al.*, 1978) was used to determine cross-reactivity of the dog, cat and human enzymes. Serial dilutions of goat anti-(human β -galactosidase) serum were performed in 10 mM-phosphate buffer, pH 7.0, containing 0.1 M-NaCl plus 0.02% NaN_3 as indicated in the legend to Fig. 2. Partially purified dog β -galactosidase (concanavalin A-Sepharose 4B effluent) and highly purified dog β -galactosidase (thiogalactoside-affinity-column eluate) were used as sources of dog β -galactosidase. Cat and human liver acid β -galactosidases, obtained as described previously (Frost *et al.*, 1978; Holmes & O'Brien, 1979), were adjusted to the same activity in solution as the dog enzyme, and serial dilutions of goat anti-(human β -galactosidase) serum were added to each preparation. The samples were kept at 4°C for 17 h, after which 10 μ l of *Staphylococcus A* protein (1 mg/ml) was added; then each sample was centrifuged at 10 000 g for 15 min, and the supernatants were assayed for β -galactosidase activity as described by Norden *et al.* (1974).

Tryptic and chymotryptic peptide mapping of dog and human β -galactosidases

Peptide mapping was performed as described by Elder *et al.* (1977). Gel slices (1 mm²) were excised from Coomassie Blue stained bands in SDS/polyacrylamide gels of β -galactosidase and washed in sealed tubes on a wheel rotator with 10% acetic acid and then with 10% methanol, each for 24 h. Proteins were labelled in the gel slice for 15 min by covering the slice with 0.020 ml of a solution containing 150–250 μ Ci of Na^{125}I , 0.2 M- Na_2HPO_4 adjusted to pH 7.5 with 0.2 M- NaH_2PO_4 , and 1 mg of chloramine-T/ml (McConahey & Dixon, 1966). The slices were rinsed with $3 \times 2 \mu$ l of water, covered with 0.1 ml of a 0.1 mg/ml solution of trypsin or chymotrypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) in 0.4% NH_4HCO_3 , and then digested in sealed tubes for 16 h at 37°C. After digestion, peptides were eluted with 0.1 ml of water, and eluates were freeze-dried to dryness, dissolved in 10% acetic acid, and samples containing 250 000 c.p.m. were spotted on to 10 cm² pre-coated cellulose thin-layer plates (American Scientific Products, Irvine, CA, U.S.A.) without fluorescent indicator. The peptides were separated by electrophoresis for 15 min at 1000 V in the first dimension and by ascending chromatography for 4 h in the second dimension as described by Kennel (1976). The resolved peptides were located by autoradiography as described by

Table 1. *Purification of dog liver acid β -galactosidase*

See the text for details of purification procedure.

Purification step	Protein (mg)	Activity (μ mol/min)	Specific activity (μ mol/min per mg)	Purification (fold)	Recovery (%)
Homogenate	226 923	590	0.0026	1.00	100
Supernatant	105 840	472	0.0044	1.69	80
Concanavalin A-Sepharose	602	214	0.335	129	36
1-thio- β -D-galactopyranoside	3.2	80	25.0	9615	14

Elder *et al.* (1977), by using Kodak XRP-1 rapid-processing X-ray film. Film exposure averaged 16 h at -70°C ; Dupont Hi-Plus intensifying screens were used to enhance peptide-spot development.

Results and discussion

The purification scheme for dog liver acid β -galactosidase is shown in Table 1. Activity in dog liver is about half of that in human liver. In a typical preparation, the fractionation scheme gave 3.2 mg of acid β -galactosidase from 1 kg of liver tissue, with a purification of 9615-fold and a recovery of 14%. Electrophoresis of this preparation on SDS/polyacrylamide slab gels gave a major band, visually estimated at about 95% of the protein, of 60 000 mol.wt., and several minor bands with molecular weights of 92 000, 55 000 and 21 000 (Fig. 1). Two of the minor bands, of mol.wts. 21 000 and 32 000, could represent the 'protective factor' and 'neuraminidase subunit' proteins, which have been reported to co-purify with acid β -galactosidase from other species (D'Azzo *et al.*, 1982; Verheijen *et al.*, 1982). However, they were present in only trace proportions in our preparations, and we do not believe them to be significant here. The final specific activities of the most purified preparations of the dog enzyme with the 4-methylumbelliferyl substrate averaged 25 μ mol cleaved/min per mg of protein. This value is somewhat lower than that for the purified human enzyme (45.5), but higher than that for purified cat acid β -galactosidase (18.6).

Results of kinetic studies on highly purified β -galactosidases (estimated at greater than 95% purity by polyacrylamide-gel electrophoresis) from each species are given in Table 2. They demonstrate that the dog enzyme had a K_m for 4-methylumbelliferyl β -D-galactopyranoside similar to that of the human and cat enzymes, but a lower K_m for *p*-nitrophenyl β -D-galactopyranoside than the other two enzymes.

After denaturation and electrophoresis in SDS, the dog enzyme migrated slightly faster than the human enzyme on polyacrylamide gels, with a molecular weight estimated at 60 000. The same pattern was obtained when the sample was electro-

Table 2. *Kinetic comparisons of purified β -galactosidases*

Dog β -galactosidase purified on an affinity column (about 95% pure; see Fig. 1) was dialysed against 100 vol. of buffer (10 mM-sodium phosphate, pH 6.0, containing 100 mM-NaCl and 0.02% NaN_3) to remove inhibitors and assayed at 37°C in 50 mM-sodium phosphate buffer, pH 4.5, containing 100 mM-NaCl, 0.02% bovine serum albumin and 0.02% NaN_3 . Assays were conducted at 37°C over a 7 min period (ten duplicate time points) at seven substrate concentrations between 0.25 and 0.60 mM for 4-methylumbelliferyl β -D-galactopyranoside (4MU) and 0.1–1.0 mM for *p*-nitrophenyl β -D-galactopyranoside (pNP). Error of duplicates was $\pm 5\%$, and linearity with time was obtained for each concentration. Kinetic constants were plotted and calculated by the method of Lineweaver & Burk (1934). Kinetic constants for the cat and human enzyme are those published previously from this laboratory by using the same method of analysis.

Species	K_m (mM)		k_{cat} (μ mol/min per mg of protein)
	4MU	pNP	4MU
Dog	0.19	0.26	25.0
Cat	0.14	0.62	18.8
Human	0.27	1.24	45.5

phoresed in SDS directly or treated with 2-mercaptoethanol, with or without prior heating, before electrophoresis. We have previously determined by gel filtration that, similar to the human acid β -galactosidase, the native dog enzyme occurs predominantly in two forms, a multimer of mol.wt. 420 000 and a dimer of mol.wt. 120 000 (Rittmann *et al.*, 1980).

When partially purified or highly purified dog acid β -galactosidase preparations were made to react with anti-(human acid β -galactosidase) antiserum, the dog enzyme was immunoprecipitated (Fig. 2). At the same enzyme activity, about 8 times more antibody was required to precipitate the dog enzyme than for the human enzyme. Under the same conditions the cat enzyme was not precipitated.

Amino acid analysis of human β -galactosidase gave 45 lysine plus arginine residues (Frost *et al.*,

Fig. 1. Polyacrylamide-gel electrophoresis of dog β -galactosidase

Purified enzyme (60 μ g) was subjected to slab-gel electrophoresis on 12.5% gels run in SDS as described in the text and stained with Coomassie Blue. Molecular weights ($\times 10^{-3}$) of standard proteins are indicated in the margin. Contaminants of mol.wts. 92000, 32000 and 21000 were faintly visible on the original gel, in addition to the major band of mol.wt. 60000.

Fig. 2. Immunoprecipitation of purified acid β -galactosidases

Goat anti-(human β -galactosidase) serum was diluted with buffer (10 mM-sodium phosphate, pH 6.0, plus 100 mM-NaCl) as indicated and 50 μ l of antibody was added to 50 μ l of purified β -galactosidase in the same buffer (plus 1 mg of bovine serum albumin/ml). After 17 h incubation at 4°C, 10 μ l of *Staphylococcus A* protein (1 mg/ml) was added and, after 15 min incubation, the samples were centrifuged at 2000g for 10 min. A sample of each supernatant was assayed for β -galactosidase activity. Samples of β -galactosidases were the highly purified preparations from each species.

present in the digests. Some 30 chymotryptic peptides were observed from β -galactosidase, but, since at least 8 of the 22 amino acids commonly found in polypeptide chains can be released by chymotryptic cleavage with varying degrees of efficiency (Smyth, 1967), we did not attempt to estimate the expected number of peptides from β -galactosidase after cleavage by chymotrypsin.

Two-dimensional tryptic and chymotryptic maps of the human and dog enzymes were very similar. The tryptic maps of human and dog enzymes (Figs. 3a and 3b) were superimposable, with the exception that four peptides from the dog enzyme had a different migration rate from the human ones (arrowed peptides, Fig. 3a), but these four still appeared to migrate in the same area as the corresponding human peptides. The two-dimensional

1978), which should yield 50 peptides after cleavage by trypsin. After such cleavage we found 26 and 25 peptides respectively from human and dog β -galactosidase (Figs. 3a and 3b), indicating that about 50% of the peptides from human and dog enzymes were

Fig. 3. Two-dimensional maps of ^{125}I -labelled polypeptides from dog and human liver acid β -galactosidase. Tryptic peptides from dog (a) and human (b) and chymotryptic peptides from dog (c) and human (d) enzymes were separated by electrophoresis in the first dimension (from right to left on each map) and by chromatography in the second dimension (from bottom to top on each map).

chymotryptic maps of human and dog enzyme (Figs. 3c and 3d) were also superimposable, except that the dog enzyme had one more peptide than the human one (arrowed peptide, Fig. 3c). Since

chymotryptic cleavage of polypeptides results in smaller peptides (Smyth, 1967), a greater tendency towards peptide homology is usually observed with chymotryptic than with tryptic generated peptides.

The extra peptide in the dog chymotryptic map may result from an asymmetric cleavage of the one differing tryptic peptide.

Tryptic and chymotryptic maps of *E. coli* β -galactosidase (results not shown) were also obtained and compared with those from the dog and human enzyme. None of the peptides from the *E. coli* enzyme overlapped with those from the dog or human enzymes.

Tryptic maps of β -galactosidase from four different control human subjects were compared, searching for electrophoretic polymorphisms, and to determine the variability of the mapping patterns. The maps from the four subjects were indistinguishable from one another.

The data indicate considerable structural homology between dog and human acid β -galactosidase and imply that the human and dog structural genes for β -galactosidase have been conserved during evolution. This is in contrast with acid β -galactosidases isolated from other species, including the bacterial, cat and mouse enzymes (Tomino & Meisler, 1975), which do not cross-react with the human enzyme. Our data indicate that the structural genes coding for human and dog acid β -galactosidases are structurally similar. For this reason canine G_{M1} gangliosidosis appears to be an excellent animal model for the disease in man.

We thank Dr. George Greaney for his help with kinetic studies. This work was supported by a grant from the Gould Family Foundation, and by NIH grants NS08682, GM16665, GM17702 to J. S. O'Brien and CA11683 to N. O. Kaplan.

References

- D'Azzo, A. D., Hoogeveen, A., Reuser, A. J. J., Robinson, D. & Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **19**, 4535–4539
- Elder, J. H., Jensen, F. C., Bryant, M. L. & Lerner, R. A. (1977) *Nature (London)* **267**, 23–28
- Frost, R. G., Holmes, E. W., Norden, A. G. W. & O'Brien, J. S. (1978) *Biochem. J.* **175**, 181–188
- Holmes, E. W. & O'Brien, J. S. (1979) *Biochemistry* **18**, 952–958
- Kennel, S. J. (1976) *J. Biol. Chem.* **251**, 6197–6204
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McConahey, P. J. & Dixon, F. J. (1966) *Int. Arch. Allergy Appl. Immunol.* **29**, 185–189
- Norden, A. G. W., Tennant, L. L. & O'Brien, J. S. (1974) *J. Biol. Chem.* **249**, 7969–7976
- O'Brien, J. S. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S., eds.), 4th edn., pp. 840–865, McGraw-Hill, New York
- Read, D. H., Harrington, D. D., Keenan, T. W. & Hinsman, E. J. (1976) *Science* **194**, 442–445
- Rittmann, L. S., Tennant, L. L. & O'Brien, J. S. (1980) *Am. J. Hum. Genet.* **32**, 880–889
- Smyth, D. G. (1967) *Methods Enzymol.* **2**, 214–230
- Tomino, S. & Meisler, M. (1975) *J. Biol. Chem.* **250**, 7752–7758
- Verheijen, F., Brossmer, R. & Galjaard, H. (1982) *Biochem. Biophys. Res. Commun.* **108**, 868–875
- Warner, T. G. & O'Brien, J. S. (1982) *J. Biol. Chem.* **257**, 224–232
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412